Biochimica et Biophysica Acta 1778 (2008) 1398–1406

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Inhibition of *Bufo arenarum* oocyte maturation induced by cholesterol depletion by methyl-β-cyclodextrin. Role of low-density caveolae-like membranes

Jorgelina Buschiazzo, Ida C. Bonini, Telma S. Alonso *

Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur – Consejo Nacional de Investigaciones Científicas y Técnicas, B8000FWB Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 12 September 2007
Received in revised form 4 March 2008
Accepted 10 March 2008
Available online 18 March 2008

Keywords:
Amphibian oocytes
Meiotic maturation
Caveolae
Src kinases
Cholesterol
Phospholipids

ABSTRACT

The invaginated structure of caveolae seems to provide an optimal environment for hormone binding leading to oocyte meiotic maturation. We conducted a quantitative analysis of lipids and proteins of detergent-free low-density membranes isolated from *Bufo arenarum* oocytes and we modulated cellular cholesterol to further understand how these domains perform their regulatory functions in the amphibian system. Light membranes derive from the plasma membrane as suggested by the enrichment in the activity of 5′nucleotidase. Lipid analysis by chromatography techniques revealed that this fraction is enriched in phosphatidylserine and cholesterol and that it evidences an important level of sphingomyelin. The finding of a single 21 kDa caveolin in light membranes indicates the presence of caveolae-like structures in *B. arenarum* oocytes. In support of this finding, c-Src is significantly associated to this fraction. Cholesterol content of oocytes treated with methyl-β-cyclodextrin (MβCD) decreased when compared to control oocytes. Drug treatment inhibited meiotic maturation in a dose-dependent manner and affected the localization of caveolin and c-Src among membrane fractions. Repletion of cholesterol showed a recovery of the ability of MβCD-treated oocytes to mature, particularly at the 25 mM concentration in which reversibility was close to the control level. Results highlight the importance of caveolae-like microdomains for maturation signaling in *Bufo* oocytes.

1. Introduction

Membrane rafts are described as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [1,2]. Caveolae are small, low-density plasma membrane invaginations which were firstly described by Palade in 1953 [3] and they are the only membrane microdomains that can be identified morphologically. Unlike lipid rafts (noninvaginated microdomains), caveolae are characterized by the presence of the cholesterol-binding protein caveolin-1 [4] which seems to be responsible for the stabilization of their invaginated structure [5]. This protein therefore serves as a specific marker for this subcellular compartment. Caveolin is a 21–24 kDa integral membrane protein which was firstly identified as a substrate for tyrosine kinase $pp60^{src}$ [6]. It has been reported to bind fatty acids [7] and it also seems to have a role in regulating intracellular free cholesterol distribution [8].

Tight interactions between cholesterol and sphingolipids lead to the formation of a more ordered lipid domain with respect to the surrounding bulk membrane, which is resistant to solubilization in non-ionic detergents [9,10]. Solubilization with cold Triton X-100 is the classical method used to obtain low-density detergent-resistant membranes (DRM) [10]. This methodology, which involves the extraction of membranes in the cold to minimize enzymatic degradation and uses detergent, could lead to phase separation and to artificial aggregation of rafts components [11]. One approach to avoid this, concerns the study of lipid rafts/caveolae at physiological temperatures and in the absence of detergents. In this respect, methods for the detection of heterogeneity in cell membranes, including fluorescence resonance energy transfer (FRET) [12] and single-particle tracking (SPT) [13], are useful tools to better learn about the size and lifetime of rafts, on the one hand, and about the dynamics and function of these microdomains in living cells, on the other. Detergent-free methods have also been developed to isolate lipid rafts/caveolae [14–16]. The importance of detergent-free methods lies in that they are unlikely either to induce membrane fusion or to selectively extract lipids and

* Corresponding author. Camino La Carrindanga km. 7, C.C. 857, B8000FWB Bahía Blanca, Argentina. Tel.: +54 291 4861201; fax: +54 291 4861200.
E-mail address: tealonso@criba.edu.ar (T.S. Alonso).

Abbreviations: ANOVA, analysis of variance; Chol, cholesterol; DPG, diphosphatidylglycerol; DRM, detergent-resistant membranes; ECL, enhanced chemiluminescence; EH, extra heavy membranes; ERK, extracellular signal-regulated kinase; FRET, fluorescence resonance energy transfer; GPI, glycosylphosphatidylinositol; GVBD, germinal vesicle breakdown; H, heavy membranes; HDM, high-density membrane; HRP, horseradish peroxidase; L, light membranes; LDM, low-density membrane; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MβCD, methyl-β-cyclodextrin; Pi, inorganic phosphorus; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; PR, progesterone receptor; PVDF, polyvinylidene difluoride; SDS- PAGE, SDS-polyacrylamide gel electrophoresis; SM, sphingomyelin; SPT, single-particle tracking; TH, total homogenate; TLC, thin-layer chromatography

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.03.004

generate rafts domains that do not exist in the intact cell. Rafts prepared by these methods are more likely to reproduce the in vivo composition of membrane microdomains.

Signaling molecules and transmembrane proteins have also been shown to be enriched in caveolae. They include heterotrimeric G-proteins [16,17], Src family kinases [16], Ras [16,18], adenylyl cyclase [19], protein kinase C [20], platelet-derived growth factor receptors, phosphatidylinositol-3 kinase, phospholipase C, and even phosphoinositides [21]. This selective localization of signaling molecules appears to facilitate signal transduction and/or play a role in physiological responses in hormone-sensitive cells such as the amphibian oocyte system.

Sato and co-workers have isolated DRM from unfertilized metaphase II-arrested *Xenopus* eggs providing evidence for the involvement of these microdomains in tyrosine kinase signaling during fertilization [22–26]. It has been also reported that *Xenopus* oocyte low-density membranes are associated with a caveolin-like protein [18]. The steroid hormone progesterone induces the resumption of meiosis in the amphibian oocyte through a nongenomic mechanism termed meiotic maturation. The initial action of progesterone in *Rana* oocytes at its plasma membrane receptor triggers a series of enzyme activations that not only modify membrane fluidity [27] but also release a cascade of lipid messengers, such as ceramide [28] and multiple diacylglycerol species [29,30]. The invaginated structure of caveolae seems to provide an optimal environment for hormone binding leading to meiotic maturation. However, the molecular bases and the mechanisms of the possible caveolar microdomain involvement in maturation signal transduction pathways have not been fully elucidated to date.

In the present study, a quantitative analysis of lipids and proteins of detergent-free low-density membranes isolated from *Bufo arenarum* oocytes and the biochemical identification of caveolae-like microdomains were performed as a prerequisite to further understand how these domains carry out their regulatory functions. Methyl-β-cyclodextrin (MβCD) was thus used for cellular cholesterol modulation in order to assess membrane raft involvement in progesterone-induced oocyte maturation.

2. Materials and methods

2.1. Experimental system

Mature females of the toad *B. arenarum* Hensel, 1867 were collected in Bahía Blanca, Argentina, during the hibernation period (winter animals) and maintained in a moist chamber until use. The ovarian tissue was surgically removed and transferred to ND 96 solution (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES; pH 7.4; Sigma, St. Louis, MO). Only those ovaries containing a large population of full-grown oocytes were used. Full-grown prophase-arrested oocytes, labelled as stage V according to Valdéz Toledo and Pisanó [31], were isolated with watchmaker's forceps under a stereotaxic microscope.

2.2. Methyl-β-cyclodextrin treatment

To deplete cellular cholesterol, full-grown ovarian oocytes were treated at different concentrations (0–50 mM) of methyl-β-cyclodextrin (Sigma) at room temperature (25 °C) during 60 min as described by Sato et al. [25]. To assess the reversibility of cholesterol depletion in MβCD-treated oocytes, cholesterol repletion was performed for 60 min at room temperature using cholesterol/MβCD complexes prepared according to the method of Christian et al. [32]. Briefly, cholesterol in chloroform:methanol (1:1, v/v) was completely dried under a stream of nitrogen. An MβCD aqueous solution at the adequate concentration was subsequently added to the dried material. The mixture was clarified by vigorous mixing and bath-sonication, and incubated in a rotating water bath at 37 °C overnight.

2.3. Induction of oocyte maturation

Maturation was induced by incubation of full-grown ovarian oocytes in ND 96 solution with progesterone (Sigma) 5 μg/ml final concentration for 7h, while control oocytes were incubated in ND 96 solution without progesterone for the same period of time. The criterion used for a successful induction of meiosis reinitiation was the verification of the germinal vesicle breakdown (GVBD) ascertained by dissection of heat-fixed oocytes.

All experiments were carried out with oocytes from clutches in which it was firstly checked that GVBD did occur with a frequency higher than 95% after progesterone treatment. Spontaneous nuclear membrane breakdown was never observed in progesterone-untreated *B. arenarum* oocytes.

2.4. Subcellular fractionation

Two different detergent-free methods were assayed in order to obtain membrane fractions from ovarian oocytes. Samples of oocytes were homogenized in 500 mM sodium carbonate, pH 11.0 and ultracentrifuged on a discontinuous sucrose gradient as described by Song et al. [16]. Alternatively, membrane fractions were isolated according to a protocol assessed for *Xenopus* oocytes [14]. Two hundred oocytes were lysed in ice-cold TNE buffer (10 mM Tris–HCl, 150 mM NaCl, and 5 mM EDTA; pH 7.4) with protease inhibitors [10 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. Oocyte homogenates were sonicated for 1 min in a cold bath at 50 Hz, 240 V, and 125 W (Branson B-220, Smithkline Co.). After 3 h ultracentrifugation on a discontinuous sucrose gradient in a SW41 Beckman rotor at 100,000 *g* and 4°C using a Beckman Coulter Optima L-90K Ultracentrifuge, three membrane fractions were isolated. Each band was collected and diluted at least four-fold with TNE buffer, and centrifuged at 200,000 *g* for 30 min, at 4 °C. Membrane pellets were either resuspended in electrophoresis buffer or extracted with chloroform:methanol (2:1) for lipid measurements.

The activity of 5′nucleotidase as an enzyme marker of plasma membrane was tested according to Widnell and Unkeless [33].

2.5. Electrophoresis and Western blotting analysis

The proteins contained in sucrose gradient fractions were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) [12.5% acrylamide] following the method of Laemmli [34]. Proteins in the gel were visualized by Coomassie staining or transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Life Science Group, Hercules, SA) for 1 h. The blot was stained with 0.5% Ponceau S in 1% acetic acid to visualize proteins or subjected to immunoblotting. Membranes were blocked overnight at 4 °C with T-TBS buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20] supplemented with 0.5% bovine serum albumin and 5% nonfat dry milk. Immunoblot detection of caveolin-like proteins and c-Src protein was performed using a 1/1000 dilution of polyclonal rabbit anti-human caveolin p21 (Transduction Laboratories, Lexington, KY) or mouse monoclonal Ig G_{2a} anti-human c-Src p60 (Santa Cruz Biotechnology, CA) at room temperature for 2 h and a 1/5000 dilution of goat anti-rabbit conjugated to horseradish peroxidase (HRP) (Transduction Laboratories, Lexington, KY) or goat anti-mouse IgG conjugated to HRP (Santa Cruz Biotechnology, CA) as secondary antibodies, at room temperature for 2 h. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) Plus Western Blotting kit (GE Healthcare UK Limited, UK).

2.6. Lipid analysis

Lipids from membrane fractions and total homogenates were extracted with chloroform:methanol (2:1, v/v) as described by Folch et al. [35]. Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel H (Merck, Germany) according to Rouser et al. [36]. Phospholipid spots were located by iodine vapors, the silica was scraped off and phosphorus was quantified after digestion with perchloric acid. Total phospholipids were measured from a lipid extract [36].

Lipids extracted according to Folch et al. [35] were dried under nitrogen stream and an aliquot was resuspended in isopropyl alcohol to determine total cholesterol by an enzymatic assay (Wiener Laboratories, Rosario, Argentina). In this procedure, 2 ml of Working Reagent (6000 U/l fungal lipase, 60 U/l cholesterol oxidase, 400 U/l peroxidase, 1.25 mmol/l 4-aminophenazone, 2.75 mmol/l phenol, pH 7.4) was combined with 20 μl of isopropyl alcohol extract and incubated at room temperature (25 °C) for 30 min. The absorbance was measured in a spectrophotometer at 505 nm and compared to a standard.

During all the procedures (lipid extraction, solvent evaporation, TLC spotting, and drying), the lipids were kept in a nitrogen atmosphere. All organic solvents were of analytical grade.

2.7. Other analytical methods

Proteins were determined using the DC Protein Assay (Bio-Rad Life Science Group, Hercules, SA). Protein assays on each of the isolated membrane fractions were performed prior to lipid extraction.

2.8. Statistical analysis

Statistical analysis was carried out using SPSS 11.0 (Inc., Chicago, IL). Analysis of variance (ANOVA) was used to determine differences among mean values which were also compared using Bonferroni *t* statistic. Student's *t* test was used to establish differences with respect to controls. In all cases, the values represent the mean ± standard deviation of the total number of samples indicated in each legend.

3. Results

3.1. Detergent-free isolation of lipid microdomains by different methods

The purification of caveolin-rich membrane domains which replaces Triton X-100 with sodium carbonate buffer and involves sonication to disrupt cellular membranes was carried out according to Song et al. [16]. Using whole oocytes as starting material after overnight ultracentrifugation on a discontinuous sucrose gradient, low-density membrane (LDM) and high-density membrane (HDM) fractions were obtained. The visible and milky LDM band, which is restricted to the 5–35% sucrose interface, included fractions 3, 4 and 5 of the gradient while the second visible band corresponding to HDM fraction (35–45% sucrose interface) included fractions 7, 8 and 9. Below fraction 9, the 45% sucrose portion was yellow but transparent.

The proteins contained in sucrose gradient fractions were resolved by SDS-PAGE. Coomassie staining showed the noteworthy presence, all along the gradient, of two bands with molecular weights coincident with yolk proteins (Fig. 1A). These bands were also detected with Ponceau S staining (Fig. 1B). In addition, differential bands were observed mainly between LDM and HDM (Fig. 1A). In amphibians, yolk proteins are a reserve of nutrients for developing embryos and they are stored in yolk platelets, the most abundant organelles of the oocyte. These proteins are 111–121 kDa lipovitellin 1, 30.5–34 kDa lipovitellin 2, and 33 kDa phosvitin, and are produced by the processing of vitellogenin in oocytes [37]. Interestingly, immunogold electron microscopy showed that the G-protein α_s subunit is present in the plasma membrane and yolk platelet membranes from *Xenopus* oocytes, forming patches [38] which seem to resemble membrane rafts.

The considerable presence of yolk proteins in these sucrose gradient fractions interfered with antibodies leading to cross-reactions. In addition, it is worthy of note that although LDM isolated by this procedure contained caveolin, other cellular proteins could be solubilized by sodium carbonate [16,39]. In agreement with this observation, it has also been reported that yolk proteins can be solubilized with salt concentrations higher than the physiological level [37].

To overcome these difficulties, an alternative detergent-free method was assayed [14]. Three membrane fractions were isolated from whole oocytes by ultracentrifugation on a discontinuous sucrose gradient: light (L) (10–22.5% sucrose interface), heavy (H) (22.5–35% sucrose interface) and extra heavy membranes (EH) (35–40% sucrose interface) (Fig. 2). These three floated bands were milky and white, and they became thinner as density increased. The sucrose layers among the conspicuous bands were clear, and, below the EH fraction, the 40% sucrose portion was always yellow. The bottom area of the centrifuge tube showed a bulky and dark pigmented pellet. In these membrane fractions the amount of yolk proteins was substantially lower (see Fig. 4A) and then, this procedure became more appropriate to obviate the above-mentioned interferences.

3.2. Activity of 5′Nucleotidase

The activity of 5′nucleotidase indicated that the fraction corresponding to L derives from the plasma membrane (Table 1). The specific activity of the enzyme in L was 6.11-fold higher than in the whole oocyte (total homogenate) while in H, the enrichment was half of that found in L. No differences were found between EH and the total homogenate, as expected.

3.3. Analysis of lipid content

A quantitative analysis of lipids from L, H and EH was carried out as a prerequisite to analyze mainly how low-density membranes perform their regulatory functions in the amphibian oocyte system. The phospholipid composition of the different membrane fractions is shown in Fig. 3. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipids in all fractions analyzed. Light membrane fraction is enriched in phosphatidylserine (PS) with respect to H and EH and it is enriched in PC, PE, sphingomyelin (SM), and phosphatidylinositol (PI) compared to EH. Similar levels of the latter phospholipids were found in the H fraction. In contrast, EH are enriched in cardiolipin or diphosphatidylglycerol (DPG), a specific component of the inner mitochondrial membrane. Regarding the PC/SM ratio, L register the lowest value (4.4) with respect to H and EH fractions (4.8 and 9.5, respectively). Phosphatidylcholine relative level was similar in all cases (~46% of total phospholipids). This difference was therefore mainly due to the increase in SM. Low amounts of lysophosphatidylcholine and lysophosphatidylethanolamine were found in all fractions. Lysophospholipids regulate a wide array of biological processes, for example, lysophosphatidylcholine induces

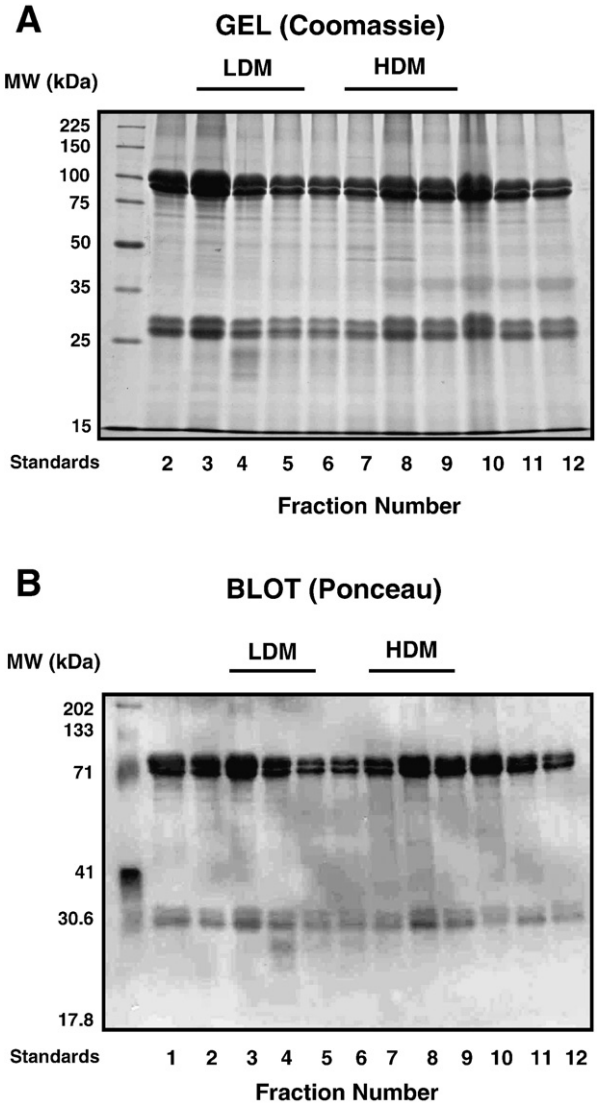

Fig. 1. Electrophoretic profiles of proteins contained in sucrose gradient fractions obtained from the whole oocyte in the absence of detergent as described by Song et al. [21]. Proteins were separated by SDS-PAGE on a 12.5% gel. (A) Proteins were visualized by Coomassie staining. (B) An equivalent gel was electroblot transferred to a PVDF membrane and visualized by staining with 0.5% Ponceau S in 1% acetic acid. Numbers to the left of each panel indicate the apparent molecular weights of commercial standards that were resolved in the left lane [A: Broad Range (Promega); B: Kaleidoscope Prestained Standards (Bio-Rad)]. LDM, low-density membrane; HDM, high-density membrane.

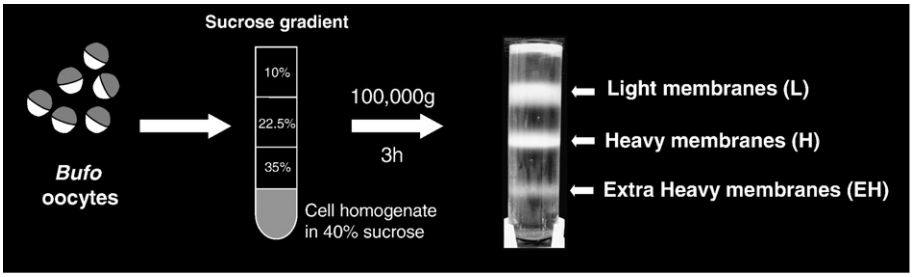

Fig. 2. Membrane fractions isolated according to Luria et al. [19] from a detergent-free oocyte homogenate by ultracentrifugation on a discontinuous sucrose gradient. Two hundred oocytes were lysed in ice-cold TNE buffer with protease inhibitors. Oocyte homogenates were sonicated for 1 min in a cold bath and were added to an equal volume of 80% sucrose to give a final equivalent density of 40% sucrose. They were then overlaid with 3 ml each of sucrose at concentrations of 35, 22.5, and 10%.

signaling responses that lead to extracellular signal-regulated kinase (ERK) activation and cell migration [40].

The amount of total phospholipids and cholesterol is shown in Table 2. Light membranes show a higher content of total phospholipids and they are enriched in cholesterol with respect to the other two heavy fractions. In L, cholesterol makes up ~49% of the total lipids measured and the cholesterol/phospholipid ratio is close to 1 (Fig. 3 and Table 1). On the other hand, SM accounts for approximately 10.4% of the total phospholipids quantified (Fig. 3). The high level of cholesterol and SM is characteristic of lipid rafts.

For comparative reasons, cortices containing plasma membrane/vitelline envelope complex were isolated by manual microdissection as described by Sadler and Maller [41]. The ratio of cholesterol to total phospholipids is 0.57 in plasma membrane cortices. Similarly, plasma membrane from *Xenopus* oocytes showed a cholesterol/phospholipid ratio of 0.51 [42].

3.4. Protein composition of membrane fractions

Regarding protein content, L show the lowest level of total proteins while H and EH have 3- and 8-fold more proteins, respectively (Table 1). Light membranes isolated following this procedure represent 0.065% of the total protein amount of one oocyte and 5.25% of one plasma membrane obtained by microdissection (4.0±0.8 μg protein). Using the same dissection method, Sadler and Maller [41] measured 5 μg of proteins per cortice obtained from *Xenopus* oocytes. The protein content and 5′nucleotidase activity were measured in the entire gradient, including interface membrane layers and aqueous phases (data not shown). In the aqueous phases, the protein amount was negligible. Below the EH (fraction 9), the 40% sucrose portion evidenced a higher protein level. The vast majority of the oocyte protein remains in the high-density region near the bottom of the gradient, as expected. It is important to remark that the enzyme marker activity was registered only in gradient fractions equivalent to L and H.

The proteins contained in sucrose gradient fractions were resolved by SDS-PAGE on 12.5% gels and they were characterized by Coomassie staining (Fig. 4A). Different profiles were observed among membrane fractions. One-dimensional electrophoresis showed bands with apparent molecular weights of ~225 kDa, ~35 kDa and two bands below 25 kDa that are enriched in L compared to H. In addition, a band of ~150 kDa in H is not present in L. Extra heavy membranes showed a distinctive protein profile. In order to determine the presence of caveolin-like proteins in *B. arenarum* oocytes, membrane fractions were subjected to immunoblot using a polyclonal antibody against caveolin p21 (Fig. 4B). A single 21 kDa caveolin was clearly enriched in L, thus indicating the presence of caveolae-like structures in *B. arenarum* oocytes. In addition, a triad of caveolin-like proteins with apparent molecular weights ranging between 30 and 40 kDa was detected in H and EH. Only the band of the triad with intermediate molecular weight was evidenced in L.

Src family kinases partition into DRM and are used as raft markers. Using a monoclonal antibody against c-Src, a single 60 kDa band was highly concentrated in the lightest fractions of the gradient (Fig. 4C).

Table 1
Protein distribution and activity of 5′Nucleotidase in total homogenate and membrane fractions obtained from *Bufo arenarum* oocytes [a]

	Protein[b] (mg × 10^{-3}/oocyte)	5′Nucleotidase activity[c] (nmol Pi/mg protein/min)	Nucleotidase activity/TH Nucleotidase activity
Total homogenate	325.00±34.16	3.29±0.47 a	1
Light membranes	0.21±0.08	20.09±2.56 b	6.11
Heavy membranes	0.62±0.15	10.52±1.42 c	3.20
Extra Heavy membranes	1.68±0.23	2.78±0.44 a	0.84

[a] Subcellular fractions were obtained as described in "Materials and methods". TH: total homogenate; Pi: inorganic phosphorus.
[b] Protein data are mean values±SD of four samples for total homogenates and eight samples for membrane fractions.
[c] The activity of 5′nucleotidase was measured according to Widnell and Unkeless [38] and it is represented as mean values±SD of seven samples for total homogenates and three independent samples for membrane fractions. Mean values of 5′nucleotidase activity were compared using Bonferroni *t* statistic. Letters (a–c) indicate significant differences ($p<0.01$) among the cases analyzed.

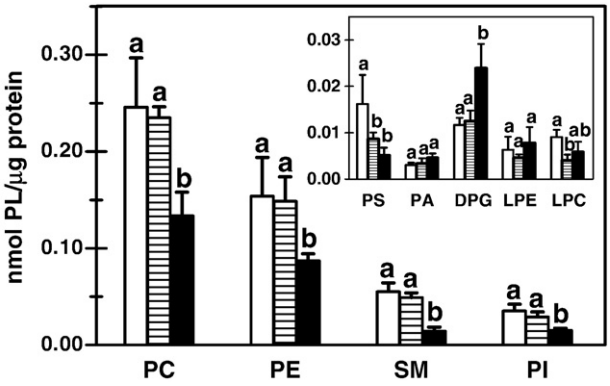

Fig. 3. Content of phospholipids in membrane fractions from *B. arenarum* oocytes. Phospholipid phosphorus was measured according to Rouser et al. [41]. Results are shown as nmol of phospholipid per mg protein and they are mean values±SD from four samples. Mean values were compared using Bonferroni *t* statistic. Letters (a–b) indicate significant differences ($p<0.05$) among the membrane fractions in each phospholipid analyzed. (□) Light membranes; (▤) Heavy membranes; (■) Extra Heavy membranes; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SM: sphingomyelin; PI: phosphatidylinositol; PS: phosphatidylserine; PA: phosphatidic acid; DPG: diphosphatidylglycerol; LPE: lysophosphatidylethanolamine; LPC: lysophosphatidylcholine; PL: phospholipid.

Table 2
Cholesterol levels and total phospholipids measured in total homogenate and membrane fractions obtained from *Bufo arenarum* oocytes[a]

	Cholesterol[b] (nmol Chol/μg protein)	Total phospholipids[b] (nmol PL/μg protein)	Chol/PL Ratio
Total homogenate	0.10±0.01 a	0.14±0.02 a	0.71
Light membranes	0.52±0.06 b	0.56±0.05 b	0.93
Heavy membranes	0.35±0.03 c	0.42±0.11 c	0.83
Extra Heavy membranes	0.13±0.04 a	0.20±0.05 a	0.65

[a] Subcellular fractions were obtained as described in "Materials and methods". Chol: cholesterol; PL: phospholipid.
[b] Total cholesterol was determined by an enzymatic assay (Wiener Laboratories, Rosario, Argentina) and total phospholipid phosphorus was measured according to Rouser et al. [41]. Data are mean values±SD of seven samples for total homogenates and four samples for membrane fractions. Mean values were compared using Bonferroni *t* statistic. Letters (a–c) indicate significant differences ($p<0.05$) among the cases analyzed within lipid groups.

3.5. Effect of cholesterol depletion and repletion on progesterone-induced oocyte maturation

In order to evaluate the possible involvement of membrane rafts in progesterone-induced oocyte maturation, we used methyl-β-cyclodextrin, the cholesterol binding drug, to deplete cellular cholesterol. If membrane order is essential for the maturation signaling, then the possible disruption of membrane microdomains by MβCD should inhibit the steroid-induced response. Oocytes were incubated at different concentrations of MβCD (5–25–50 mM) for 60 min at 25 °C, the amphibian physiological temperature. Cholesterol content decreased about 18% and 21% when oocytes were treated with 25 mM and 50 mM of MβCD, respectively. No changes were found in oocytes treated at 5 mM of MβCD (Fig. 5A). A concomitant cholesterol decrease (20%) was observed in L obtained from MβCD-treated oocytes (Fig. 5B).

To assess the reversibility of the MβCD-induced cholesterol depletion in *Bufo* oocytes, cholesterol repletion was performed using cholesterol/MβCD complexes. In both concentrations analyzed, cholesterol was incorporated into *Bufo* oocytes in a reversible manner reaching the level of the control oocytes (Fig. 5A).

When MβCD-treated oocytes were incubated with progesterone to induce meiotic maturation, GVBD underwent a significant decrease compared to control oocytes (Fig. 6). We found that MβCD at 25 mM reduced the GVBD response by 23% and with 50 mM MβCD the inhibition was higher (41%) indicating that inhibition of maturation was dose-dependent. Cholesterol repletion showed a recovery of the ability to mature of MβCD-treated oocytes. At 25 mM MβCD the reversibility was near the control suggesting that at this concentration the drug does not result toxic for oocytes. Oocytes treated with 50 mM MβCD not only failed to recover the control oocyte ability to mature but also became fragile and sometimes wrinkled.

Under our experimental conditions, MβCD-treated oocytes that were not incubated with progesterone did not show GVBD indicating that the drug alone does not induce the maturation response.

3.6. Effect of MβCD treatment on membrane fractions

To evaluate if MβCD treatment affects the localization of the raft markers, caveolin and c-Src, we isolate L, H and EH fractions from oocytes treated at 25 mM MβCD. As it was mentioned above, at this concentration treated oocytes are still able to recover the ability to mature like control oocytes.

Protein profile of membrane fractions evaluated by Coomassie staining was not affected by MβCD treatment (data not shown). Membrane fractions analyzed by immunoblotting evidenced that now caveolin did not localize to L of MβCD-treated oocytes but to the heavy fractions (Fig. 7). As to Src, the drug treatment caused a redistribution of this signal molecule which still localized to L but showed an increase in the H fraction and a mild expression in EH. These results are compatible with a disorganization of membrane rafts produced by MβCD.

4. Discussion

Recent studies have suggested that lipid rafts are heterogeneous in terms of their lipid and protein composition and that they therefore represent a collection of related domains with similar physical properties [for review, see 44]. Detergent-free methods do not involve the solubilization of membranes and isolate membrane rafts on the basis of their low-density. In order to compare the properties of low-density membrane fractions obtained with and without detergent extraction, Luria et al. [14] have isolated DRM and light membranes

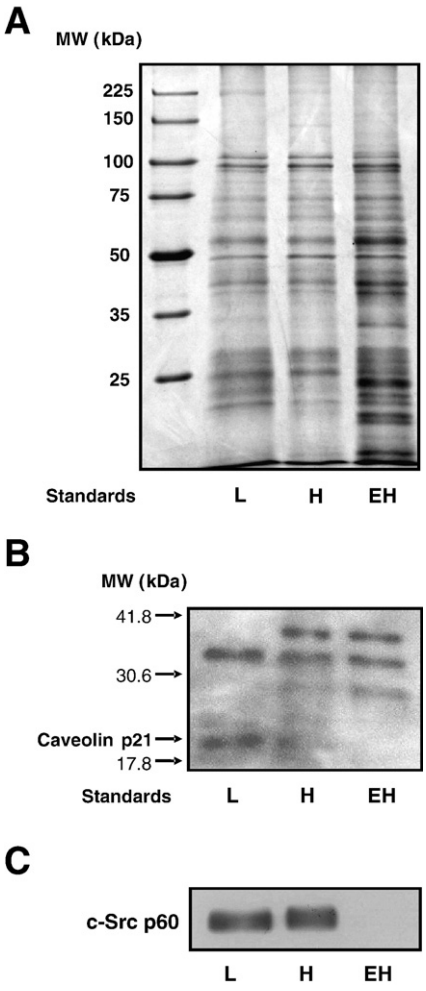

Fig. 4. Proteins of membrane fractions isolated from a detergent-free oocyte homogenate by ultracentrifugation on a discontinuous sucrose gradient [19]. Proteins were separated by SDS-PAGE on a 12.5% gel. (A) Proteins were visualized by Coomassie staining (25 μg prot/lane). (B) Immunoblot detection (20 μg prot/lane) of caveolin-like proteins was performed using a 1/1000 dilution of polyclonal rabbit anti-human caveolin p21 and a 1/5000 dilution of goat anti-rabbit conjugated to HRP as secondary antibody. (C) Immunoblot detection (10 μg prot/lane) of c-Src was performed using a 1/1000 dilution of mouse monoclonal Ig G_{2a} anti-human c-Src p60 and a 1/5000 dilution of goat anti-mouse IgG conjugated to HRP as secondary antibody. Images were analyzed using ImageJ [43]. Results are representative of three different experiments. Numbers to the left of each panel indicate the apparent molecular weights of commercial standards that were resolved in the left lane [A: Broad Range (Promega); B: Kaleidoscope Prestained Standards (Bio-Rad)]. L, Light membranes; H, Heavy membranes; EH, Extra Heavy membranes.

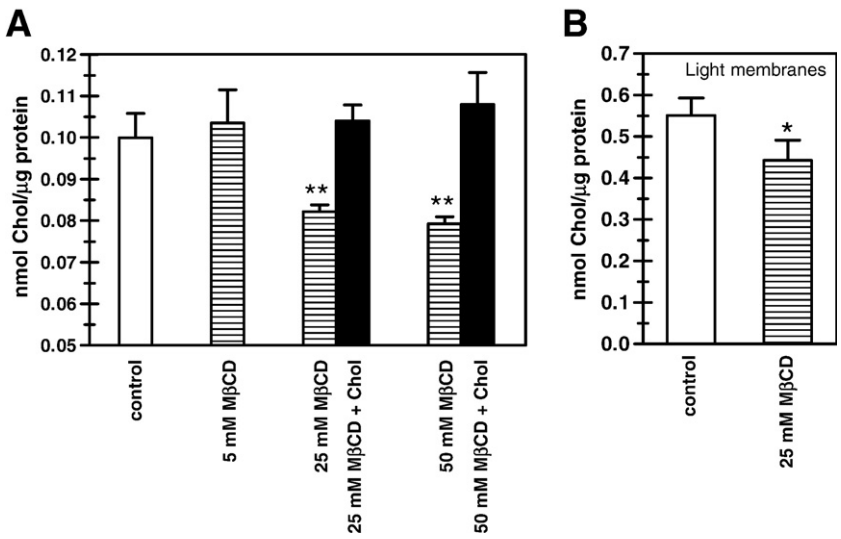

Fig. 5. Cholesterol depletion and repletion of *B. arenarum* oocytes. (A) Full-grown ovarian oocytes were incubated in the absence or the presence of increasing concentrations of MβCD for 60 min at 25°C to remove cellular cholesterol. Cholesterol repletion was carried out incubating MβCD-treated oocytes with cholesterol/MβCD mixtures. (B) Cholesterol content of light membranes isolated from oocytes untreated and treated with 25 mM of MβCD. Data represent the mean±standard deviation of three independent trials using 30 oocytes for each condition. Asterisks indicate significant differences with respect to control (*$p<0.05$ or **$p<0.01$). MβCD, methyl-β-cyclodextrin; Chol, cholesterol.

from mature *Xenopus laevis* eggs. Based on physical and biochemical studies, they found that the detergent-free light membrane fraction is similar to DRM.

Although amphibian oocytes are an excellent model for the study of membrane function, the lipid microdomain involvement in signal transduction pathways has not been thoroughly studied to date.

In the present work, we have isolated, in the absence of detergents, a light membrane fraction from *B. arenarum* oocytes physiologically arrested in G2 of meiosis I. A relevant finding concerns the presence of a 21 kDa caveolin clearly enriched in the L fraction indicating the existence of caveolae-like structures in *B. arenarum* oocytes. Consistent with this, Src kinase, commonly used as a raft marker, is significantly associated to L.

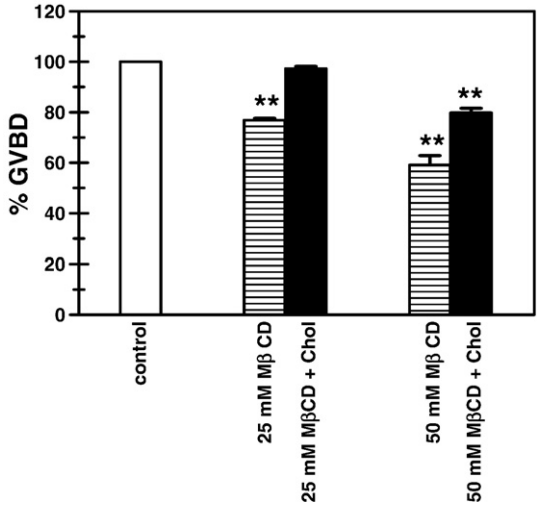

Fig. 6. Effect of cholesterol depletion and repletion on progesterone-induced oocyte maturation. Full-grown ovarian oocytes were incubated in the absence or the presence of increasing concentrations of MβCD for 60 min at 25°C to remove cellular cholesterol. Cholesterol repletion was carried out incubating MβCD-treated oocytes with cholesterol/MβCD mixtures. After depletion/repletion treatment, oocytes were washed and incubated with progesterone to induce meiotic maturation. Successful induction of meiosis reinitiation was verified by the germinal vesicle breakdown. Data represent the mean±standard deviation of three independent trials using 30 oocytes for each condition. Asterisks indicate significant differences with respect to control ($p<0.01$). MβCD, methyl-β-cyclodextrin; Chol, cholesterol; GVBD, germinal vesicle breakdown.

Fractions L and H derive from the plasma membrane as suggested by the enrichment in the activity of 5′nucleotidase, a classical plasma membrane enzyme marker (Table 1). However, the specific activity of the enzyme in H is half of that found in L. In *X. laevis*, cell surface biotinylation and the presence of several plasma membrane markers showed that both light and heavy membranes derive from the plasma membrane while the extra heavy fraction is originated mainly from intracellular components [14]. Similar results were found in thymocytes using also a discontinuous sucrose gradient isolation procedure. The activity of enzyme markers, including 5′nucleotidase, permitted to conclude that the two lightest fractions originate from the thymocyte plasma membrane [45,46]. It is also important to note that glycosylphosphatidylinositol (GPI)-anchored 5′nucleotidase has been found mainly in caveolae [47,48]. According to this finding, 5′nucleotidase not only functions as a plasma membrane enzyme marker but also indicates the possibility that caveolar microdomains are present in a specific cell type. Anderson [49] proposed a model for intercellular signaling by caveolar patches in which, caveolae could function as storage sites for messenger molecules such as adenosine, and in which, the stimulation of the cell releases the messenger into the intercellular space.

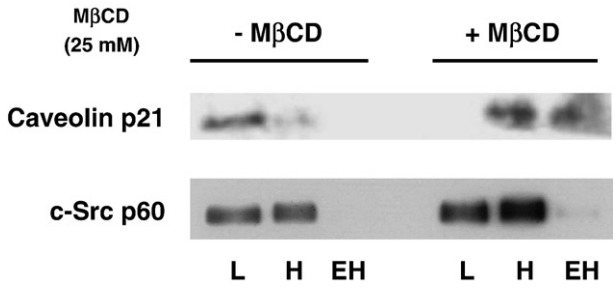

Fig. 7. Effect of MβCD treatment on membrane fractions isolated from *B. arenarum* oocytes. Membrane fractions were isolated from oocytes untreated and treated with 25 mM of MβCD. Immunoblot detection (20 µg prot/lane) of caveolin-like proteins was performed using a 1/1000 dilution of polyclonal rabbit anti-human caveolin p21 and a 1/5000 dilution of goat anti-rabbit conjugated to HRP as secondary antibody. Detection (10 µg prot/lane) of c-Src was performed using a 1/1000 dilution of mouse monoclonal Ig G_{2a} anti-human c-Src p60 and a 1/5000 dilution of goat anti-mouse IgG conjugated to HRP as secondary antibody. Results are representative of three different experiments. MβCD, methyl-β-cyclodextrin; L, Light membranes; H, Heavy membranes; EH, Extra Heavy membranes.

Lipid determinations performed in our work evidence that PC, PE, SM, PI and cholesterol contents in L and H fractions are higher than in EH which is clearly enriched in the mitochondrial lipid marker DPG (Fig. 3 and Table 2). Heavy fraction seems to derive from the plasma membrane but differs from L in the cholesterol and PS contents, which are significantly lower in H with respect to L, as well as in the above-mentioned relatively lower enzyme activity and in the absence of an intensely reactive 21 kDa caveolin band. Even when c-Src was found to be present in H, it was clearly associated to L. Altogether, this comparative analysis implies that the three membrane fractions represent different entities: a fraction enriched mainly in low-density caveolae-like domains derived from the plasma membrane (L); another fraction enriched in noncaveolar plasma membrane (H); and, a third fraction enriched in intracellular components (EH).

On the other hand, results indicate that PC and PE, as major phospholipids of biological membranes, are also the most abundant in L, H and EH from *Bufo* oocytes. Light membranes are enriched in PS with respect to the two heavy fractions. In neurons, LDM isolated in the absence of detergent represented ~5–10% of the plasma membrane protein (this percentage is similar to the ~5% observed in our study), and they were enriched in cholesterol, SM, PC, and PE with respect to heavy membranes [50]. It has been reported that there are differences between the lipid composition of DRM and nondetergent rafts isolated from the same cells [51]. Cholesterol and choline phospholipids, PC and SM, are most often located in the outer leaflet of the membrane whereas PE and the acidic phospholipids tend to be found mainly in the inner leaflet of the membrane. Detergent treatment of membranes may selectively extract the exofacial leaflet of rafts while detergent-free procedures give rise to membrane preparations with a balance of inner and outer leaflet lipids [51]. Our results show that, using this detergent-free protocol, these caveolae-like membranes evidence high levels of the typical outer leaflet lipids as well as of PE and the acidic phospholipid PS, thus indicating, in agreement with previous reports, that rafts are probably bilayer structures [51].

The acidic phospholipids represent approximately 10% of the total phospholipids in L mainly due to the PS amount. A PS-binding protein, which is a substrate of protein kinase C (PKC), is involved in the PKC binding to caveolae [52]. Moreover, multiple PKC isoenzymes were found to be enriched in caveolae [53]. In addition, annexins are soluble proteins that bind to negatively charged phospholipids such as PS and they associate with membrane domains of distinct lipid composition [54]. As a result, PS seems to be important for the function of membrane rafts.

Light membranes from *Bufo* oocytes are enriched in cholesterol and they show an important level of SM, in agreement with the typical lipid composition of a membrane raft. About 28% of the plasma membrane cholesterol is located in L and both lipids make up ~55% of the total lipids measured. Sphingomyelin interacts very tightly with cholesterol and this serves as the basis for raft formation [55]. Moreover, SM hydrolysis by sphingomyelinases generates ceramides that have been implicated as signal transduction mediators in certain cell types. It has been reported that lipid microdomains are involved in the intercellular movement of ceramide from mammalian oocytes into cumulus cells [56]. In addition, sphingomyelinases have been detected in membrane rafts [57].

Results concerning the protein content of the sucrose gradient fractions evidenced that the protein level is higher in the heavy fractions. Similar results were found in free-detergent membrane fractions from *Xenopus* eggs [14]. Interestingly, the protein profile showed differential bands among membrane fractions. The identification of distinctive protein bands associated to L could be very useful to better define these membranes biochemically and functionally. In this respect, we are able to obtain sufficient amount of protein for identification by mass spectrometry.

The biochemical identification of caveolae-like structures in *B. arenarum* oocytes was achieved by the detection of a 21 kDa caveolin-like protein (Fig. 4B). In *X. laevis* oocytes a 21 kDa caveolin-like band was detected in LDM from cortical membranes. However, a complete gradient profile was not shown [18]. Furthermore, caveolin-1 was detected in DRM from *Xenopus* eggs but it was also distributed among the high-density fractions of the gradient [25]. On the other hand, immunoblot detection of a signal molecule such as c-Src associated to this light membrane fraction suggests that caveolae-like structures may play a role in signaling mechanisms operating in *Bufo* oocytes. In *Xenopus* eggs, xSrc is localized to the low density detergent-insoluble membranes and is involved in fertilization [25].

Higher molecular weight caveolin-like bands detected mainly in the heavy membranes from *Bufo* oocytes were also found in *Xenopus* oocytes [18]. Caveolin exists as a high molecular mass homo-oligomer of ~350 kDa with ~14–16 caveolin monomers per oligomer [58]. It has been reported that caveolin homo-oligomers can self-associate in vitro [58] and that caveolin-1 can interact with caveolin-2 to generate a caveolar coat [59]. Thus, the presence of higher molecular weight caveolin-like bands in *Bufo* membrane fractions suggests that the interaction among caveolin monomers generates caveolin hetero- or homodimers.

The importance of nongenomic actions of steroid hormone receptors and their ability to associate with the plasma membrane as well as to activate rapid signaling pathways are now considered important features of these receptors. Progesterone induces amphibian oocytes, quiescent in prophase I of meiosis, to re-enter the meiotic cycle. However, the nature of the progesterone receptor (PR) is still elusive. A new PR with the binding site located in the α1-subunit of the membrane Na/K-ATPase has been recently reported in *Rana pipiens* oocytes [60].

To date, the signal transduction pathways of progesterone action at the plasma membrane remain unclear. In the present work, we examined the functional and structural importance of low-density caveolae-like membranes during progesterone-induced oocyte maturation analyzed in different cholesterol environments. Interestingly, when *Bufo* oocytes were treated with the drug MβCD to deplete cholesterol, meiotic maturation was inhibited in a dose-dependent manner (Fig. 6). Even when changes in cholesterol content were limited to a decrease of about 18% and 21% at the two concentrations analyzed (Fig. 5A), we found a significant effect on meiosis reinitiation. In addition, a significant cholesterol decrease was found in L fraction isolated from MβCD-treated oocytes (Fig. 5B). In *Xenopus* oocytes, cholesterol depletion stimulated oocyte meiotic maturation in spite of the fact that the authors originally hypothesized that MβCD treatment would inhibit the steroid-induced response [61].

It is important to note that the degree of cholesterol depletion may differ significantly between cell types even when comparable MβCD concentrations and exposure times are applied [for review, see 62]. In mammalian cell types, cholesterol depletion is efficiently carried out at 37 °C but this temperature may damage amphibian oocytes. In our study, the MβCD treatment was performed at the amphibian physiological temperature. Such temperature treatment condition could be responsible for reducing cholesterol removal. In addition, amphibian oocytes are surrounded by the glycoprotein vitelline envelope which may reduce the contact between the drug and the plasma membrane. Similarly, in *Xenopus* oocytes an increased resistance to cholesterol depletion was reported [61]. In spite of these amphibian oocyte characteristics, the treatment with MβCD changed the localization of c-Src and caveolin among membrane fractions (Fig. 7). Altogether, these results suggest that oocyte caveolae-like domains are structurally and functionally affected by MβCD treatment.

Several studies have reported that cholesterol depletion leads to the disorganization of detergent resistant/low-density membrane fractions. Cholesterol depletion induced loss of caveolae and inhibition of chemotaxis in neurons [63]. Using steady state fluorescence anisotropy, it was demonstrated that cholesterol removal results in an increase in the fluidity of some of the lipid microdomains of sperm

membranes [64]. Furthermore, the treatment with MβCD inhibited fertilization possibly by dispersing sea urchin egg rafts [65].

To assess the specificity of MβCD effects, cholesterol-matched controls were used as previously suggested [62]. The exposure to MβCD saturated with cholesterol may not maintain normal levels of cholesterol but it could significantly increase cholesterol above the control level [62]. As it occurs in cholesterol depletion, cholesterol enrichment may affect cellular functions. In our study, only at 25 mM of MβCD, treated oocytes were able to recover the ability to mature as the control oocytes even when cholesterol repletion was close to the normal level at both concentrations analyzed (Figs. 5 and 6).

The presence of the raft markers caveolin and c-Src in oocyte membrane microdomains, can recreate in part, signaling events in meiotic maturation. Src kinases play a pivotal role as membrane-attached molecular switches that link a variety of extracellular cues to critical intracellular signaling pathways. On the other hand, caveolin-1, as a scaffolding protein, organizes signaling molecules in the caveolae enabling regulation of physiological responses. It has been recently demonstrated that human PR interacts with Src and mediates rapid activation of Src and downstream MAPK (Erk-1/-2) [66]. In *Xenopus* oocytes, Src kinase accelerated oocyte maturation through the MAPK pathway [67]. Interestingly, it has been reported that estrogen receptors localize to caveolae/rafts [68,69] and that estrogen receptor α associates with caveolin-1 [for review, see 70].

Our results of this study demonstrate that *B. arenarum* oocyte maturation is inhibited by cholesterol depletion induced by MβCD. In addition, a disturbance in caveolae-like microdomains was revealed through the relocation of raft markers after drug treatment.

Our research provides lines of evidence regarding the presence of caveolae-like microdomains in *B. arenarum* oocytes and highlights the importance of caveolae-like microdomains for the maturation signaling in *Bufo* oocytes.

Acknowledgements

We are grateful to Dr. María F. Pediconi for her critical reading of the manuscript and to Translator Viviana Soler for her technical assistance in controlling the use of the English language. This research was supported by grants from *Universidad Nacional del Sur* (PGI, 24/B105) and *Comisión de Investigaciones Científicas de la Provincia de Buenos Aires* (CIC, 183/505).

References

[1] L.J. Pike, Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function, J. Lipid Res. 47 (2006) 1597–1598.
[2] S. Mayor, A. Viola, R.V. Stan, M.A. del Pozo, Flying kites on slippery slopes at Keystone, Symposium on Lipid Rafts and Cell Function, EMBO Rep. 7 (2006) 1089–1093.
[3] G.E. Palade, Fine structure of blood capillaries, J. Appl. Phys. 24 (1953) 1424.
[4] M. Murata, J. Peranen, R. Schreiner, F. Wieland, T.V. Kurzchalia, K. Simons, VIP21/caveolin is a cholesterol-binding protein, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10339–10343.
[5] P.U. Le, G. Guay, Y. Altschuler, I.R. Nabi, Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum, J. Biol. Chem. 277 (2002) 3371–3379.
[6] J.R. Glenney, L. Zokas, Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton, J. Cell Biol. 108 (1989) 2401–2408.
[7] B.L. Trigatti, R.G. Anderson, G.E. Gerber, Identification of caveolin-1 as a fatty acid binding protein, Biochem. Biophys. Res. Commun. 255 (1999) 34–39.
[8] S. Le Lay, E. Hajduch, M.R. Lindsay, X. Le Liepvre, C. Thiele, P. Ferre, R.G. Parton, T. Kurzchalia, K. Simons, I. Dugail, Cholesterol-induced caveolin targeting to lipid droplets in adipocytes: a role for caveolar endocytosis, Traffic 7 (2006) 549–561.
[9] D.A. Brown, J.K. Rose, Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface, Cell 68 (1992) 533–544.
[10] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221–17224.
[11] H. Heerklotz, H. Szadkowska, T. Anderson, J. Seelig, The sensitivity of lipid domains to small perturbations demonstrated by the effect of Triton, J. Mol. Biol. 329 (2003) 793–799.
[12] D.A. Zacharias, J.D. Violin, A.C. Newton, R.Y. Tsien, Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells, Science 296 (2002) 913–916.
[13] C. Dietrich, B. Yang, T. Fujiwara, A. Kusumi, K. Jacobson, Relationship of lipid rafts to transient confinement zones detected by single particle tracking, Biophys. J. 82 (2002) 274–284.
[14] A. Luria, V. Vegelyte-Avery, B. Stith, N.M. Tsvetkova, W.F. Wolkers, J.H. Crowe, F. Tablin, R. Nuccitelli, Detergent-free domain isolated from *Xenopus* egg plasma membrane with properties similar to those of detergent-resistant membranes, Biochemistry 131 (2002) 13189–13197.
[15] E.J. Smart, Y.S. Ying, C. Mineo, R.G. Anderson, A detergent-free method for purifying caveolae membrane from tissue culture cells, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10104–10108.
[16] K.S. Song, S. Li, T. Okamoto, L.A. Quilliam, M. Sargiacomo, M.P. Lisanti, Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains, J. Biol. Chem. 271 (1996) 9690–9697.
[17] P. Oh, J.E. Schnitzer, Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default, Mol. Biol. Cell 12 (2001) 685–698.
[18] S.E. Sadler, Low-density caveolae-like membrane from *Xenopus laevis* oocytes is enriched in Ras, J. Cell. Biochem. 83 (2001) 21–32.
[19] R.S. Ostrom, C. Gregorian, R.M. Drenan, Y. Xiang, J.W. Regan, P.A. Insel, Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase, J. Biol. Chem. 276 (2001) 42063–42069.
[20] E.J. Smart, Y.S. Ying, R.G. Anderson, Hormonal regulation of caveolae internalization, J. Cell Biol. 131 (1995) 929–938.
[21] L.J. Pike, L. Casey, Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains, J. Biol. Chem. 271 (1996) 26453–26456.
[22] A.K. Mahbub Hasan, K. Sato, K. Sakakibara, Z. Ou, T. Iwasaki, Y. Ueda, Y. Fukami, Uroplakin III, a novel Src substrate in *Xenopus* egg rafts, is a target for sperm protease essential for fertilization, Dev. Biol. 286 (2005) 483–492.
[23] A.K. Mahbub Hasan, Z. Ou, K. Sakakibara, S. Hirahara, T. Iwasaki, K. Sato, Y. Fukami, Characterization of *Xenopus* egg membrane microdomains containing uroplakin Ib/III complex: roles of their molecular interactions for subcellular localization and signal transduction, Genes Cells 12 (2007) 251–267.
[24] K. Sakakibara, K. Sato, K. Yoshino, N. Oshiro, S. Hirahara, A.K. Mahbub Hasan, T. Iwasaki, Y. Ueda, Y. Iwao, K. Yonezawa, Y. Fukami, Molecular identification and characterization of *Xenopus* egg uroplakin III, an egg raft-associated transmembrane protein that is tyrosine-phosphorylated upon fertilization, J. Biol. Chem. 280 (2005) 15029–15037.
[25] K. Sato, T. Iwasaki, K. Ogawa, M. Konishi, A.A. Tokmakov, Y. Fukami, Low density detergent-insoluble membrane of *Xenopus* eggs: subcellular microdomain for tyrosine kinase signaling in fertilization, Development 129 (2002) 885–896.
[26] K. Sato, A.A. Tokmakov, C.L. He, M. Kurokawa, T. Iwasaki, M. Shirouzu, R.A. Fissore, S. Yokoyama, Y. Fukami, Reconstitution of Src-dependent phospholipase Cgamma phosphorylation and transient calcium release by using membrane rafts and cell-free extracts from *Xenopus* eggs, J. Biol. Chem. 278 (2003) 38413–38420.
[27] G.A. Morrill, K. Doi, A.B. Kostellow, Progesterone induces transient changes in plasma membrane fluidity of amphibian oocytes during the first meiotic division, Arch. Biochem. Biophys. 269 (1989) 690–694.
[28] G.A. Morrill, A.B. Kostellow, Progesterone release of lipid second messengers at the amphibian oocyte plasma membrane: role of ceramide in initiating the G2/M transition, Biochem. Biophys. Res. Commun. 246 (1998) 359–363.
[29] G.A. Morrill, A.B. Kostellow, Molecular species analysis of 1, 2- diacylglycerol released in response to progesterone binding to the amphibian oocyte plasma membrane, Cell. Signal. 12 (2000) 787–796.
[30] B.J. Stith, A.J. Kirkwood, E. Wohnlich, Insulin-like growth factor 1, insulin, and progesterone induce early and late increases in *Xenopus* oocyte sn-1,2-diacylglycerol levels before meiotic cell division, J. Cell. Physiol. 149 (1991) 252–259.
[31] C.L. Valdéz Toledo, A. Pisanó, Studies of oogenesis in *Bufo arenarum*, Reproducción 4 (1980) 315–330.
[32] A.E. Christian, M.P. Haynes, M.C. Phillips, G.H. Rothblat, Use of cyclodextrins for manipulating cellular cholesterol content, J. Lipid Res. 38 (1997) 2264–2272.
[33] C.C. Widnell, J.C. Unkeless, Partial purification of a lipoprotein with 5′-nucleotidase activity from membranes of rat liver cells, Proc. Natl. Acad. Sci. U. S. A. 61 (1968) 1050–1057.
[34] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[35] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[36] G. Rouser, S. Fleischer, A. Yamamoto, Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[37] N. Yoshizaki, A. Moriyama, S. Yonezawa, Purification and properties of embryonic cysteine proteinase which participates in yolk-lysis of *Xenopus laevis*, Comp. Biochem. Physiol., B Biochem. Mol. Biol. 119 (1998) 571–576.
[38] C.J. Gallo, A.R. Hand, T.L. Jones, L.A. Jaffe, Stimulation of *Xenopus* oocyte maturation by inhibition of the G-protein alpha S subunit, a component of the plasma membrane and yolk platelet membranes, J. Cell Biol. 130 (1995) 275–284.
[39] M. Sargiacomo, M. Sudol, Z. Tang, M.P. Lisanti, Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells, J. Cell Biol. 122 (1993) 789–807.

[40] L. Wang, C.G. Radu, L.V. Yang, L.A. Bentolila, M. Riedinger, O.N. Witte, Lysophosphatidylcholine-induced surface redistribution regulates signaling of the murine G protein-coupled receptor G2A, Mol. Biol. Cell 16 (2005) 2234–2247.
[41] S.E. Sadler, J.L. Maller, Progesterone inhibits adenylate cyclase in *Xenopus* oocytes. Action on the guanine nucleotide regulatory protein, J. Biol. Chem. 256 (1981) 6368–6373.
[42] J. Santiago, G.R. Guzmán, L.V. Rojas, R. Marti, G.A. Asmar-Rovira, L.F. Santana, M. McNamee, J.A. Lasalde-Dominicci, Probing the effects of membrane cholesterol in the *Torpedo californica* acetylcholine receptor and the novel lipid-exposed mutation alpha C418W in *Xenopus* oocytes, J. Biol. Chem. 276 (2001) 46523–46532.
[43] W.S. Rasband, ImageJ, U S National Institutes of Health, Bethesda, Maryland, U. S. A. (1997–2007) (URL: http://rsb.info.nih.gov/ij/).
[44] L.J. Pike, Lipid rafts: heterogeneity on the high seas, Biochem. J. 378 (2004) 281–292.
[45] A. Monneron, J. d'Alayer, Isolation of plasma and nuclear membranes of thymocytes. I. Enzymatic composition and ultrastructure, J. Cell Biol. 77 (1978) 211–231.
[46] A. Monneron, J. d'Alayer, Isolation of plasma and nuclear membranes of thymocytes. II. Biochemical composition, J. Cell Biol. 77 (1978) 232–245.
[47] F.C. Andersson, L.E. Gustafsson, Cytochemical localization of 5′-nucleotidase in the enteric ganglia and in smooth muscle cells of the guinea-pig, J. Neurocytol. 14 (1985) 551–562.
[48] A. Kittel, E. Bacsy, Ecto-ATPases and 5′-nucleotidases in the caveolae of smooth muscle. Enzyme-histochemical evidence may indicate a role for caveolae in neurotransmission, Cell Biol. Int. 18 (1994) 875–879.
[49] R.G. Anderson, Caveolae: where incoming and outgoing messengers meet, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 10909–10913.
[50] C. Wu, S. Butz, Y. Ying, R.G. Anderson, Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane, J. Biol. Chem. 272 (1997) 3554–3559.
[51] L.J. Pike, X. Han, R.W. Gross, Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study, J. Biol. Chem. 280 (2005) 26796–26804.
[52] S. Gustincich, P. Vatta, S. Goruppi, M. Wolf, S. Saccone, V.G. Della, M. Baggiolini, C. Schneider, The human serum deprivation response gene (SDPR) maps to 2q32-q33 and codes for a phosphatidylserine-binding protein, Genomics 57 (1999) 120–129.
[53] C. Mineo, Y.S. Ying, C. Chapline, S. Jaken, R.G. Anderson, Targeting of protein kinase Calpha to caveolae, J. Cell Biol. 141 (1998) 601–610.
[54] A. Draeger, S. Wray, E.B. Babiychuk, Domain architecture of the smooth-muscle plasma membrane: regulation by annexins, Biochem. J. 387 (2005) 309–314.
[55] A.E. Cremesti, F.M. Goni, R. Kolesnick, Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Lett. 531 (2002) 47–53.
[56] G.I. Perez, A. Jurisicova, T. Matikainen, T. Moriyama, M.R. Kim, Y. Takai, J.K. Pru, R.N. Kolesnick, J.L. Tilly, A central role for ceramide in the age-related acceleration of apoptosis in the female germline, FASEB J. 19 (2005) 860–862.
[57] R.J. Veldman, N. Maestre, O.M. Aduib, J.A. Medin, R. Salvayre, T. Levade, A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signaling, Biochem. J. 355 (2001) 859–868.
[58] M. Sargiacomo, P.E. Scherer, Z. Tang, E. Kubler, K.S. Song, M.C. Sanders, M.P. Lisanti, Oligomeric structure of caveolin: implications for caveolae membrane organization, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9407–9411.
[59] R. Mora, V.L. Bonilha, A. Marmorstein, P.E. Scherer, D. Brown, M.P. Lisanti, E. Rodriguez-Boulan, Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1, J. Biol. Chem. 274 (1999) 25708–25717.
[60] G.A. Morrill, A.B. Kostellow, A. Asakari, Progesterone binding to the α1-subunit of the Na/K-ATPase on the cell surface: Insights from computational modeling, Steroids 73 (2008) 27–40.
[61] S.E. Sadler, N.D. Jacobs, Stimulation of *Xenopus laevis* oocyte maturation by methyl-beta-cyclodextrin, Biol. Reprod. 70 (2004) 1685–1692.
[62] R. Zidovetzki, I. Levitan, Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies, Biochim. Biophys. Acta 1768 (2007) 1311–1324.
[63] D. Lentini, F. Guzzi, F. Pimpinelli, R. Zaninetti, A. Cassetti, S. Coco, R. Maggi, M. Parenti, Polarization of caveolins and caveolae during migration of immortalized neurons, J. Neurochem. 104 (2008) 514–523.
[64] M. Companyó, A. Iborra, J. Villaverde, P. Martínez, A. Morros, Membrane fluidity changes in goat sperm induced by cholesterol depletion using beta-cyclodextrin, Biochim. Biophys. Acta 1768 (2007) 2246–2255.
[65] R.J. Belton, N.L. Adams, K.R. Foltz, Isolation and characterization of sea urchin egg lipid rafts and their possible function during fertilization, Mol. Reprod. Dev. 59 (2001) 294–305.
[66] V. Boonyaratanakornkit, E. McGowan, L. Sherman, M.A. Mancini, B.J. Cheskis, D.P. Edwards, The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle, Mol. Endocrinol. 21 (2007) 359–375.
[67] A. Tokmakov, T. Iwasaki, S. Itakura, K. Sato, M. Shirouzu, Y. Fukami, S. Yokoyama, Regulation of src kinase activity during *Xenopus* oocyte maturation, Dev. Biol. 278 (2005) 289–300.
[68] K.L. Chambliss, I.S. Yuhanna, R.G.W. Anderson, M.E. Mendelsohn, P.W. Shaul, ERβ has nongenomic action in caveolae, Mol. Endocrinol. 16 (2002) 938–946.
[69] C. Heberden, F. Reine, B. Grosse, C. Henry, Y. Zagar, G. Chaumaz, M. Lieberherr, Detection of a raft-located estrogen receptor-like protein distinct from ERα, Int. J. Biochem. Cell Biol. 38 (2006) 376–391.
[70] E.R. Levin, Integration of the extranuclear and nuclear actions of estrogen, Mol. Endocrinol. 19 (2005) 1951–1959.